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Metabolism of Nicotine to (+)-gamma-(3-Pyridyl)-gamma-methylaminobutyric Acid

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[Contribution from the Department of Pharmacology, Medical College of Virginia]

Metabolism of Nicotine to (+)-γ-(3-Pyridyl)-γ-methylaminobutyric Acid[1]

By Herbert McKennis, Jr.,[2a] Lennox B. Turnbull and Edward R. Bowman[2b]

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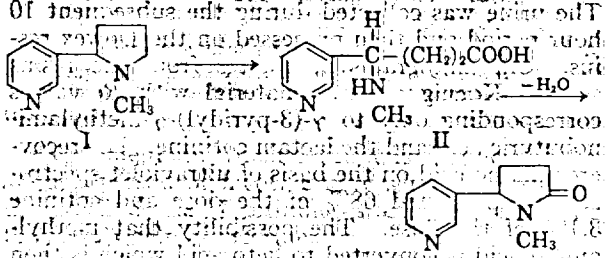
Metabolism of (−)-nicotine in the dog leads to the formation of (+)-γ-3-(pyridyl)-γ-methylaminobutyric acid and cotinine. Under conditions of physiological *p*H, γ-3-(pyridyl)-γ-methylaminobutyric acid spontaneously cyclizes to the lactam cotinine. The reverse reaction, hydrolysis of the cotinine, does not occur appreciably under comparable conditions. The evidence suggests that an enzyme system is not obligatory for formation of cotinine *per se* and that a hydrolytic enzyme is required if cotinine is a natural precursor of γ-(3-pyridyl)-γ-methylaminobutyric acid.

In a previous communication from this Laboratory[3] data were presented to show that γ-(3-pyridyl)-γ-methylaminobutyric acid arises during the metabolism of nicotine in the dog. The appearance of this acid authenticated in part a general hypothetical route for the metabolism of nicotine which has been discussed previously.[4] It is now shown that both γ-(3-pyridyl)-γ-methylaminobutyric acid and its lactam cotinine arise from nicotine under physiological conditions. The relationships between nicotine and its metabolites which are experimentally verified as a result of these findings are represented below



Following intravenous administration of (−)-nicotine (I) the urine of dogs was acidified to *p*H 2 and then placed on a Dowex 50 (H^+) column which retained all of the Koenig positive material in the urine. Elution with *N*/1 ammonia water removed the Koenig positive material (disclosed with *p*-aminobenzoic acid and cyanogen bromide) which could be separated into six zones upon paper chromatography with ammonia–ethanol–butanol. These zones, which were absent from control urine, were compared for position by cochromatography with nicotine and a number of its derivatives. The results indicated the presence of nicotine (R_f 0.86), which had previously been reported to be present in urine by many authors.[5,6] The comparison suggested also that the material at R_f 0.73 was cotinine (III) and that the material at R_f 0.15 was γ-3-pyridyl-γ-methylaminobutyric acid (II).

Chloroform extraction of the ammoniacal solution of Koenig positive compounds removed the three faster moving components (nicotine, cotinine and unknown at R_f 0.61). The aqueous residue was then placed upon Dowex 1 (OH^-). The component with R_f 0.15 was retained. Components with R_f 0.38 and R_f 0.46 were not retained on the resin and are, hence, pyridine compounds with no or only feebly acidic functions. In addition it was determined through extraction of the zones from paper chromatograms that all of the substances in ethanolic HCl had single absorption maxima at 260 mμ. This places the metabolites as a group in a class of pyridine compounds with no side chain double bonds conjugated with the pyridine ring.[7] If one assumes a common molecular extinction coefficient, it can be seen (Table I) that the five metabolites account in one animal for approximately 21% of the administered dose of nicotine (153 mg.). The greatest quantity of material (9.2%) appeared at R_f 0.15. This R_f value corresponded to that of authentic γ-(3-pyridyl)-γ-methylaminobutyric acid. Further the material on heating became chloroform soluble and changed in R_f value to that of cotinine. These findings led to an unequivocal chemical identification.

Table I

Metabolism of Nicotine (153 Mg. I. V.) by Male Dog (15.3 Kg.)

	R_f[a] value of metabolite in Dowex 50 eluate	Mole,[b] % of dose
(1)	0.15	9.21
(2)	.38	5.40
(3)	.46	0.88
(4)	.61	2.79
(5)	.73	2.84

[a] Values were determined in the ammonia–ethanol–butanol system on Whatman No. 1 paper by the descending method at room temperature. For spectroscopic investigation areas corresponding to the Koenig positive zones were localized as quenching spots in ultraviolet light. R_f value of zone 1 corresponds to γ-(3-pyridyl)-γ-methyl-aminobutyric acid and R_f value of zone 5 corresponds to cotinine simultaneously chromatographed. The influence of temperature, paper and other factors on R_f values and the homogeneity of the zones have not been systematically investigated. R_f values were generally consistent to ± 0.05 unit but occasional aberrant values have been encountered. Control urine yielded no positive zones. [b] Calculated from optical density of eluted chromatographic zones at 260 mμ (in 0.1 *M* HCl in 95% ethanol)—optical density of eluate from metabolite free paper extracted in a similar fashion, using ε 4820.[5]

(1) Presented in part at the Symposium on Tobacco Chemistry, Southeastern Regional Meeting of the American Chemical Society, Durham, North Carolina, November 15, 1957.

(2) (a) Appreciation is expressed to the Tobacco Industry Research Committee and the American Tobacco Company for support of this investigation. (b) Public Health Research Fellow of the National Heart Institute.

(3) H. McKennis, Jr., L. B. Turnbull and E. R. Bowman, This Journal, 79, 6342 (1957); Abstracts, Southeastern Regional Meeting, American Chemical Society, November 14–16, 1957, p. 23.

(4) H. McKennis, Jr., L. B. Turnbull, H. N. Wingfield, Jr., and L. J. Dewey, This Journal, 80, 1634 (1958).

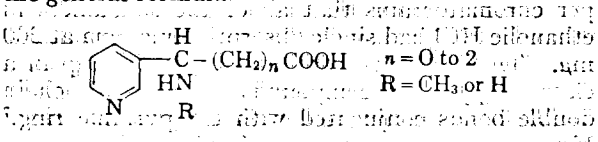
(5) A. C. Corcoran, O. M. Helmer and I. H. Page, *J. Biol. Chem.*, 129, 89 (1939); J. K. Finnegan, P. S. Larson and H. B. Haag, *J. Pharmacol. Exp. Therap.*, 91, 357 (1947).

(6) P. S. Larson and H. B. Haag, *ibid.*, 76, 240 (1942).

(7) M. L. Swain, A. Bisner, C. F. Woodward and B. A. Brice, This Journal, 71, 1341 (1949).

A Dowex 1 (OH^-) column which contained the R_f 0.15 material from the urine of six dogs was eluted with $N/1$ acetic acid. The eluate was concentrated to a brown sirup and heated to 155° under nitrogen to yield 45 mg. of oil which was soluble in chloroform. The oil yielded a crystalline picrate, m.p. 104–106°, which did not depress the melting point of authentic cotinine picrate and gave the correct analysis for C, H and N. The infrared absorption spectra of isolated and authentic picrates were identical. This established the metabolite as γ-(3-pyridyl)-γ-methylaminobutyric acid (II).

In previous studies[6,8,9] it was observed that following administration of nicotine, the urine of dogs contained material which gave a red color with cyanogen bromide without the addition of aromatic amine. Since this material could not be extracted from alkaline solution with ether and a variety of 3-pyridylalkanes were found to produce such a color if the carbon atom adjacent to the pyridine ring bore a primary or secondary amine, it was concluded that the data suggested the presence of an acid of the general formula



It was noted also that if the urine was boiled prior to reaction with cyanogen bromide the red color did not develop. *A priori*, if a single compound is involved in the reaction with cyanogen bromide, this compound is γ-(3-pyridyl)-γ-methylaminobutyric acid (n = 2, R = CH_3). The acid itself gives a direct red color with cyanogen bromide[16] and spontaneously or upon heating forms the lactam cotinine which does not give the color reaction. γ-(3-Pyridyl)-γ-aminobutyric acid (n = 2, R = H) gives a similar color reaction and forms the lactam desmethylcotinine[4] on heating. It is presumed from the current data that if γ-3-pyridyl-γ-aminobutyric acid is present in the urine it escaped detection in the procedures of isolation and chromatography employed in the present investigation. The lower homologs of the amino acids, where n = 0 to 1 and R = H or CH_3, although they may be present in the urine in small amounts, do not have properties which would readily explain the observed reactions. Where n = 0 no lactam can be formed and β-lactams, where n = 1, can be formed only with difficulty and under special conditions.[10]

Cotinine from the thermal cyclization of the metabolically formed γ-(3-pyridyl)-γ-methylaminobutyric acid displayed the same optical rotation as cotinine obtained from the oxidation of nicotine by Pinner's procedure[11] or cotinine resulting from the cyclization of γ-(3-pyridyl)-γ-methylaminobutyric acid which had been prepared by the published procedure.[4] Metabolic γ-(3-pyridyl)-γ-methylaminobutyric acid, therefore, has an approximate rotation of $[\alpha]D$ +17° and an asymmetric carbon atom with the same absolute configuration as that of (−)nicotine.[12]

Since it has been proposed[13] that γ-(3-pyridyl)-γ-methylaminobutyric acid can serve as an intermediate in the degradation of nicotine to γ-(3-pyridyl)-γ-oxobutyric acid, a bacterial metabolite of nicotine,[14,15] it was of interest to seek evidence for the keto acid in urine of nicotine-treated dogs. An examination of the paper chromatograms revealed no Koenig positive spot in the region of 0.24. If the keto acid had been present in the urine to the extent of 0.5% of the nicotine administered, the acid could have been easily detected by this procedure. The apparent lack of appreciable conversion of γ-(3-pyridyl)-γ-methylaminobutyric acid to γ-(3-pyridyl)-γ-oxobutyric acid is reaffirmed by a number of other observations. Following slow intravenous administration of 110 mg. of (+)-γ-(3-pyridyl)-γ-methylaminobutyric acid to a dog[16] the urinary output of the compound, based on the cyanogen bromide color reaction, amounted to 86%. In our studies a dog was given (+)-γ-(3-pyridyl)-γ-methylaminobutyric acid in a single intraperitoneal dose. The urine was collected during the subsequent 10 hour period and then processed on the Dowex resins. Chromatograms of eluates from the resins showed Koenig positive material with R_f values corresponding only to γ-(3-pyridyl)-γ-methylaminobutyric acid and the lactam cotinine. The recovered amino acid on the basis of ultraviolet spectroscopy represented 68% of the dose and cotinine 3.1% of the dose. The possibility that methylamino acid is converted to keto acid which is then metabolized to the methylamino acid or other substances is, of course, not eliminated by the present studies. Experiments on the metabolism of γ-(3-pyridyl)-γ-oxobutyric acid are now in progress.

γ-(3-Pyridyl)-γ-methylaminobutyric acid, in the metabolism of nicotine, is on formal grounds both a precursor and metabolite of cotinine. A limited number of experiments were conducted to establish some of the factors concerned with this relationship. It was observed frequently during the course of the experiments that older samples of urine contained increasingly larger amounts of material in the cotinine zone of chromatogams and increasingly smaller concentrations of γ-(pyridyl)-γ-methylaminobutyric acid. This spontaneous lactamization is in agreement with the previously noted instability of the compound.[4] Since the urines of dogs in most of the experiments were excreted in the region of pH 5.2,[17] consideration was given to

(8) P. S. Larson, H. B. Haag and J. K. Finnegan, *J. Pharmacol. Exp. Therap.*, **86**, 239 (1946).

(9) P. S. Larson, *Ind. Eng. Chem.*, **44**, 279 (1952).

(10) S. A. Ballard, D. D. Melstrom and C. W. Smith in "The Chemistry of Penicillin," H. T. Clarke, J. R. Johnson and R. Robinson, Editors, Princeton University Press, Princeton, N. J., 1949, p. 973; J. C. Sheehan and E. J. Corey, *Organic Reactions*, **9**, 388 (1957).

(11) A. Pinner, *Ber.*, **26**, 292 (1893).

(12) It is assumed that in the preparation of cotinine from nicotine by the bromination procedure[11] there has been no inversion in the 2-position. There have been no experimental determinations of the absolute configuration of cotinine and only one of nicotine [P. Karrer and R. Widmer, *Helv. Chim. Acta*, **8**, 364 (1925)].

(13) E. Werle, H. Schievelbein and D. Spieth, *Arzneimittel-Forsch.*, **6**, 322 (1956).

(14) E. Wada and K. Yamasaki, THIS JOURNAL, **76**, 155 (1954).

(15) T. Tabuchi, *J. Agr. Chem. Soc. Japan*, **29**, 222 (1955).

(16) F. B. Owen, Jr., and P. S. Larson, *Arch. int. Pharmacodyn.*, **115**, 402 (1958).

(17) The urinary pH influences the amount of lactamization and cotinine formation *in vivo* and *in vitro* and bears also upon the amount of materials which may be reabsorbed from the urinary tract for subsequent additional exposure to enzymatic processes. Travell (*Proc.*

the possibility that cotinine was entirely an artifact which arose during the collecting and processing of the urine. Studies (Table II) on the effect of *p*H on the stability of γ-(3-pyridyl)-γ-methylaminobutyric acid showed that within 0.5 hr. at 20° and *p*H 7 significant amounts of cotinine were formed. Even at *p*H 8.9 the amino acid in solution when heated to 100° is readily converted to cotinine. In contrast no cotinine was converted to amino acid in the *p*H range studied. These results indicate that, irrespective of initial mode of formation, some γ-(3-pyridyl)-γ-methylaminobutyric acid will serve as a precursor of cotinine in the biological systems studied. It is further concluded that any major direct conversion of cotinine to γ-(3-pyridyl)-γ-methylaminobutyric acid would depend upon the presence of an enzymatic system.

TABLE II

STABILITY OF AQUEOUS SOLUTIONS (2.86×10^{-5} *M*) OF γ-(3-PYRIDYL)-γ-METHYLAMINOBUTYRIC ACID

*p*H 0.1 *M* Na phosphate	Products after 1 hr.[a,b] 22°	100°
4.25	Acid > cotinine	acid = cotinine
6.40	Acid > cotinine	acid > cotinine
7.00	Trace cotinine	acid > cotinine
8.92	No cotinine	acid > cotinine

[a] The amount of product was estimated by paper chromatography and the Koenig reaction. [b] Cotinine ($3–4 \times 10^{-5}$ *M*) yielded no detectable amount of amino acid under these conditions.

Experiments in which cotinine is supplied to the animal instead of nicotine may serve to elucidate the possible role of cotinine as an intermediate in the metabolism of nicotine.

Acknowledgment.—The authors wish to thank Mr. Quentin S. McKennis and Mr. James Harlan for valuable assistance.

Experimental

Intravenous Administration of Nicotine to Mongrel Dogs.—Throughout an 8-hour period a solution of nicotine in 0.9% sodium chloride was infused into the femoral vein at a rate of 1 ml./min. The nicotine content of the saline was adjusted so that each animal under anesthesia received a total dose of 10 mg./kg. Urine was collected through a catheter during the infusion and during the subsequent 10-hour period.

Processing of Dog Urine for Nicotine Metabolites.—The pooled urine (1438 ml. representing 670 mg. of nicotine) was filtered through diatomaceous earth (Celite), and the *p*H was adjusted to *p*H 2 with 5 *N* HCl. The acidified urine was then placed on a Dowex 50 × −4 (H+) column (14 × 2.5 cm.). The column was washed with water (approx. 2 l.) until washings were virtually colorless. The column was then eluted with *N*/1 ammonia water (approx. 150 ml.) until no more Koenig positive material was obtained. The yellow brown solution was evaporated to dryness at room temperature. The residue was triturated with absolute ethanol (30 ml.). The alcoholic solution was filtered.[20] The filtrate was treated with decolorizing carbon and then concentrated to dryness. The residue was dissolved in 30 ml. of distilled water. The aqueous solution was extracted continuously with chloroform for 20 hr. After removal of chloroform under diminished pressure the extract weighed 118 mg. After a further treatment with decolorizing carbon the aqueous phase was adjusted to *p*H 10–11 with ammonia water and then placed on a Dowex 1 (OH) column. The effluent contained Koenig positive material which was combined with Koenig positive material obtained by washing the column with water. This fraction weighed 1025 mg. after removal of the solvent. The column was then eluted with *N*/1 acetic acid until no more Koenig positive material could be obtained. This solution upon concentration to dryness at room temperature yielded 383 mg. of brown solid (Fraction C) with Koenig positive material at R_f 0.15, corresponding to γ-(3-pyridyl)-γ-methylaminobutyric acid. All chromatograms were conducted by the descending method on Whatman No. 1 paper at room temperature with ammoniacal alcohol (1 vol. *N*/2 ammonia water, 1 vol. 95% ethanol, 4 vol. 1-butanol).

Identification of γ-(3-Pyridyl)-γ-methylaminobutyric Acid in Fraction C.—The above obtained 383 mg. of brown solid was heated to 155° in an atmosphere of nitrogen for 15 minutes and then dissolved in 15 ml. of water. After a treatment with decolorizing carbon, the clear solution was extracted with six portions (15 ml.) of chloroform. The residue, upon removal of the chloroform, weighed 43 mg. (λ_{max} 262 mμ in ethanol). In paper chromatograms the oil yielded a single Koenig positive zone corresponding to cotinine (R_f 0.73).

Cotinine Picrate.—A sample of (−)cotinine was treated with an excess of a saturated methanolic solution of picric acid. After several days in the refrigerator the crystalline residue was collected and recrystallized from 95% ethanol (m.p. 104–106°). For analysis the sample was dried at 1 mm. over KOH.

Anal. Calcd. for $C_{18}H_{15}N_9O_8$: C, 47.41; H, 3.73; N, 17.28. Found: C, 47.45; H, 3.80; N, 17.34.

Cotinine Picrate from Metabolic γ-(3-Pyridyl)-γ-methylaminobutyric Acid.—The 43-mg. sample of cotinine from the urinary methylamino acid was treated with an excess of methanolic picric acid. The precipitate after four recrystallizations from 95% ethanol weighed 21 mg. (m.p. 104–106°). Admixture with authentic cotinine picrate caused no depression of the melting point. In Nujol mulls the infrared absorption spectra of authentic and isolated samples were identical.[21]

Anal. Calcd. for $C_{18}H_{15}N_9O_8$: C, 47.41; H, 3.73; N, 17.28. Found: C, 47.49; H, 3.80; N, 17.19.

Formation of γ-(3-Pyridyl)-γ-methylaminobutyric Acid by Male Dog.—Since previous studies on nicotine metabolism were accomplished largely on female dogs, urine from a male dog (12.8 kg.) which had received nicotine as described above, was processed on Dowex 50 as described above. The solids from the chloroform extract, which contained cotinine, nicotine and unidentified metabolite(s), weighed 128 mg. Cyclization of the Dowex 1 eluate which contained γ-(3-pyridyl)-γ-methylaminobutyric acid resulted in 9.4 mg. of cotinine with extinction coefficient at 260 mμ in 0.1 *M* HCl in 95% ethanol corresponding to a calculated 9.02 mg. of cotinine. On a molar basis this represents 6.9% of the dose of nicotine. A similar experiment conducted with a female dog (14.1 kg.) resulted in the isolation of 3.6 mg. of thermally formed cotinine, identified by paper chromatography and ultraviolet spectrum, corresponding to 3.8 mg. of γ-(3-pyridyl)-γ-methylaminobutyric acid, or 2.32% of the dose of nicotine.

Metabolism of γ-(3-Pyridyl)-γ-methylaminobutyric Acid in the Dog.—An unanesthetized male mongrel dog (9.0 kg.) was given 810 mg. of γ-(3-pyridyl)-γ-methylaminobutyric acid intraperitoneally. The urine was collected in the ensuing 22-hour period. The Koenig positive fraction from the Dowex 50 column was adjusted to a volume of 50 ml. An aliquot (0.1 ml.) was chromatographed on paper. Only two Koenig positive spots (corresponding to γ-(3-pyridyl)-γ-methylaminobutyric acid and cotinine) were obtained. The Koenig positive areas were eluted with 0.1 *M* HCl in 95% ethanol. The ultraviolet absorption spectra of these

Soc. Exper. Biol. Med., **45**, 552 (1940)) noted that alkalinity favored absorption of nicotine from the bladder of the cat. Others[18,19] have considered the effect of *p*H on reabsorption in humans.

(18) J. Travell, O. Bodansky and H. Gold, *J. Pharmacol. Exp. Therap.*, **69**, 307 (1940).

(19) H. B. Haag and P. S. Larson, *ibid.*, **76**, 235 (1942).

(20) In some experiments it was found that additional Koenig positive material can be recovered from the residue by water extraction.

(21) We thank Mr. W. B. Wartman, Jr., of the Research Laboratory, American Tobacco Company, for these determinations. When the spectra were determined in KBr pellets, small differences were noted in the region above 7 μ. Since both isolated and authentic specimens behaved similarly in this regard the differences are attributed to polymorphism or possible crystal orientation effects.

indicated an excretion of 68% of the amino acid unchanged and 3.1% of the amino acid appeared as cotinine.

Thermal Lactamization of Synthetic and Metabolic γ-(3-Pyridyl)-γ-methylaminobutyric Acid.—The monohydrate of the methylamino acid[4] (100 mg.) ($[\alpha]^{22}D$ +17.2) in a Pyrex tube was heated for 0.5 hr. in an atmosphere of nitrogen at 145–150°. The oily residue was extracted with six portions of chloroform (5 ml. each). The product was dissolved in methanol and gave an optical rotation ($[\alpha]^{21.5}_{5461}$ −18.16° (*c* 4.35)).

Fraction C (303 mg.) from the pooled urine of six dogs (893 mg. of administered nicotine) was heated for 0.5 hr. at 145–150° under nitrogen. The residue then was dissolved in 5 ml. of distilled water. The solution was extracted with six portions (5 ml. each) of chloroform. Upon evaporation of the chloroform extract under diminished pressure a residue of cotinine (49.8 mg.) or 5.55% of the dose of nicotine was obtained ($[\alpha]^{30}_{5461}$ −18.77° (*c* 4.98)). In comparison a sample of cotinine prepared from nicotine by the method of Pinner[11] had a specific rotation ($[\alpha]^{30}_{5461}$ −19.85; (*c* 5.59, methanol)).

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